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MISSISSIPPI RIVER BASIN STEROL ASSAY PROJECT REPORT. COPROSTANOL, A POSITIVE MOLECULAR MARKER OF DOMESTIC AND RUN-OFF POLLUTION

STEROL ASSAY OF RAW SEWAGE, WASTEWATER PLANT EFFLUENT AND SURFACE WATERS IN THE BURLINGTON, IOWA AREA ON THE MISSISSIPPI RIVER

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Sterol Assay of Raw Sewage, Wastewater Plant Effluent and Surface Waters in the Burlington, Iowa Area on the Mississippi River

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ENVIRONMENTAL PROTECTION AGENCY
Water Quality Office
Advanced Waste Treatment Research Laboratory
Cincinnati, Ohio
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COPROSTANOL, A POSITIVE MOLECULAR MARKER OF DOMESTIC AND RUN-OFF POLLUTION

II. Sterol Assay of Raw Sewage, Wastewater Plant Effluents and Surface Waters in the Burlington, Iowa Area on the Mississippi River

Henry H. Tabak and Robert L. Bunch

Several studies have recently emphasized the merits of using coprostanol, a major human fecal sterol as a positive molecular marker of domestic pollution in addition to the standard method for enumeration of fecal coliforms (Murtaugh and Bunch, 1967, Smith and Gouron, 1969, Tabak and Bunch, 1970). Bunch and his coworkers (1967) have shown that coprostanol as well as other sterols can be removed from wastewater by an adequate secondary sewage treatment.

This study was undertaken to estimate the extent of human and warm-blooded animal fecal pollution and to pinpoint the main sources of this pollution within a 3 mile radius of Burlington, Iowa on the Mississippi River. The survey covered seven sampling points, including raw sewage to the Burlington Wastewater Treatment Plant, the effluent and surface water up and down stream from the treatment plant outfall. The sampling points and their river mileage are described in Appendix I. Each location was sampled six different times at one week intervals. The samples were iced and flown to this laboratory for sterol analysis. They were about one day old when analysed for the fecal sterol. The Burlington waste treatment plant influent and effluent are normally chlorinated. The first three sets of samples of influent and effluent came in as unneutralized samples, whereas the next three replicate sets were neutralized by sodium thiosulfate.

The analytical method used for the estimation of the coprostanol was based on the methodology which was previously developed in this laboratory (Murtaugh and Bunch, 1967) and proven effective for the recovery of sterols from wastewater as well as from a stream in the Cincinnati, Ohio area. Recently, it was applied successfully to wastewaters, wastewater treatment plant effluents, and surface waters of the Lower Main Stem Missouri. The procedure consists of hexane extraction, mild alkali-alcohol hydrolysis of esters and conjugates to free parent sterols, cleanup by thin-layer chromatography (TLC) and quantitative measurement by gas-liquid chromatography (GLC). The procedure used is fully described in Appendix II.

Simultaneously with the assay for the fecal sterol, the number of fecal coliforms were determined utilizing the membrane filter technology.

Results and Discussion

The Burlington sewage treatment plant influent and effluent samples were shown to have a significantly higher concentration of the fecal sterol than surface water samples taken at points just before the plant sewage effluent discharge area or further down the river away from the treatment plant. The decrease in concentration of coprostanol was demonstrated adequately by analysis of water samples taken further away along the river from the location of treatment plant outfall. The analytical data demonstrate no significant reduction in the concentration of coprostanol in the treated effluent from that present in raw sewage influent, thus providing evidence for inadequacy of the primary sewage treatment in removing sterols.

Table 1 summarizes the analytical data on the concentration of coprostanol in the samples from seven sampling points. The surface water samples taken from sampling point at river mileages 405.6, 404.3 and 403.4 upstream from the sewage treatment plant contained a mean concentration of coprostanol of 8, 7 and 6 μg/l respectively. The Burlington STP influent samples taken on plant site at river mileage 403.2 contained a concentration of coprostanol in the range from 245 to 394 μg/l while the treated STP effluent samples taken at the same location gave values in the range from 160 to 315 μg/l. The mean values for the influent and STP effluent were 316 and 240 μg/l respectively. The water samples taken at the STP mixing zone sampling point at river mileage 403.15 downstream from the plant gave values in the range from 28 to 35 μg/l with a mean of 31 μg/l. The range values of coprostanol concentration in surface water samples taken from sampling point at river mileage 403.0 downstream from the plant were 4 to 12 μg/l with an average of 9 μg/l.

A resume on the enumeration of fecal coliforms in the treated sewage effluents, raw sewage influents, and surface water samples from the Mississippi River is given in Table 2. The microbiological data substantiate the studies on the extent of fecal pollution as determined by the fecal sterol assay. Surface river samples upstream from the treatment plant and downstream from the plant sewage outfall show a progressively lower density of fecal coliforms. Since the same trend was demonstrated for the concentration of coprostanol in the influent, effluent and surface river water samples, there seems to be a definite and significant correlation between concentration of the fecal sterol and the degree of fecal pollution. This relationship is graphically shown in Figure 1.

On an average, a hog, a cow or a human being would be expected to excrete 800 to 1,000 mg of coprostanol per day. If this is diluted with 100 gallons of wastewater, the expected concentration would be 210 to 250 μg/l in raw wastewater. The results as compiled in Table 1 show that the treatment plant effluent coprostanol concentration falls within this amount, and the raw sewage coprostanol concentration slightly exceeds this value. The Mississippi River water samples were approximately 10-fold lower in the fecal sterol concentration than the Missouri River water samples (Tabak and Bunch, 1970) and almost equal in value to those taken below an outfall of a poorly run secondary treatment plant in the Cincinnati area (Murtaugh and Bunch, 1967).

Summary

Raw sewage influent, sewage treatment plant effluent, and surface river water samples in the 3-mile radius of Burlington, Iowa on the Mississippi River were analyzed for the presence of the fecal sterol, coprostanol.

Significant variations in the amount of coprostanol in the effluents and surface waters at sampling points further away from sewage effluent outfalls were clearly demonstrated and these show a correlation to the density of fecal coliforms at the same locations.

Insignificant reduction in the concentration of coprostanol in treated effluents from that present in raw sewage influents points to the inability of the primary sewage treatment in removing coprostanol and other sterols.

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Tabak, H. H. and Bunch, R. L. 1970. Missouri River Basin Sterol Assay Project Report. Coprostanol, a Positive Marker of Domestic and Run-off Pollution. Sterol assay of wastewater effluents and surface waters of the Lower Main Stem Missouri. Intradepartmental Report.

TABLE I

Coprostan-3-beta-ol Assay Data

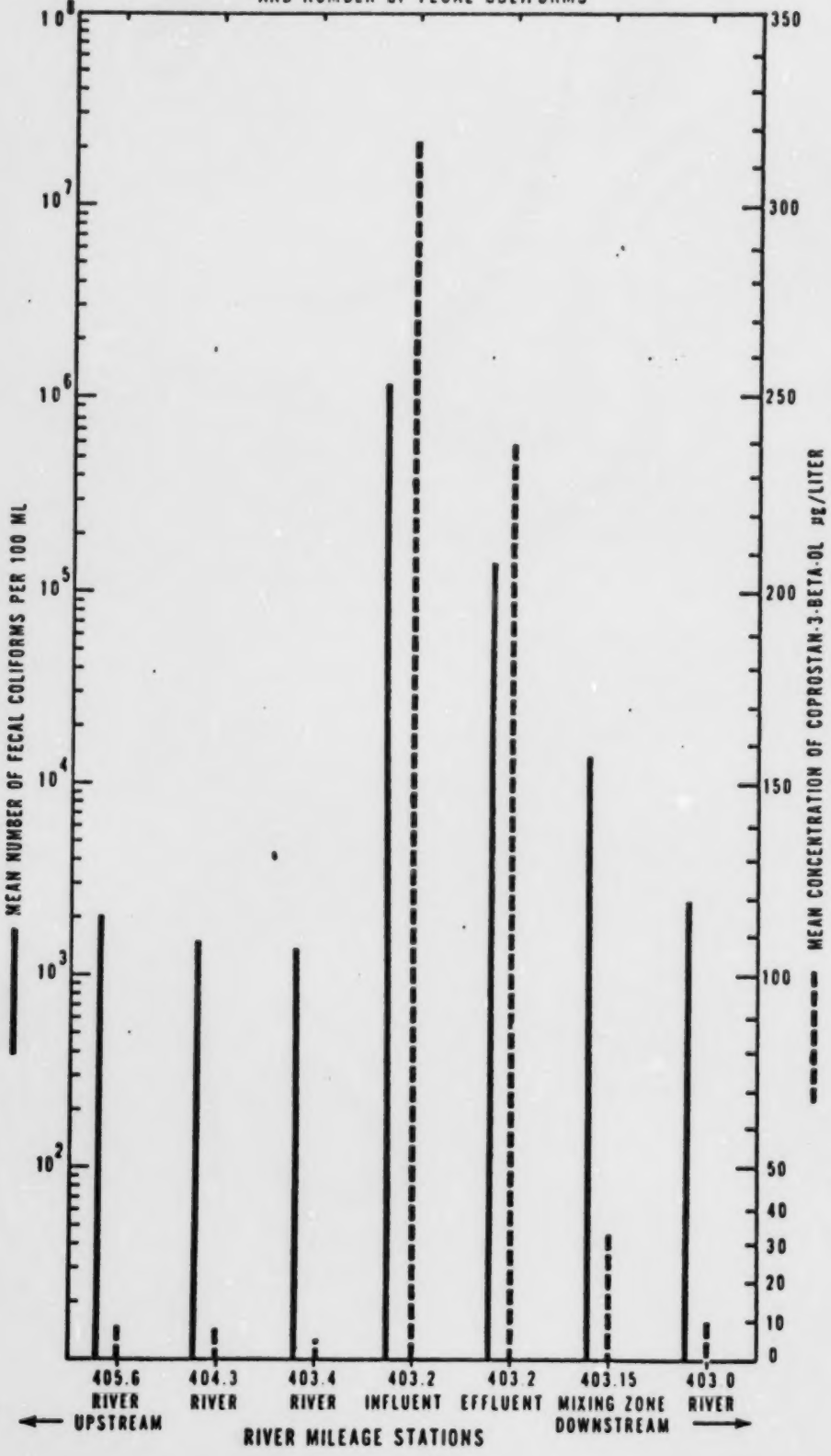
	SAMPLING POINT	RIVER MILEAGE	CONCENTRATION OF COPROSTANOL IN µg/liter 8-27	9-2	9-9	9-16	9-24	9-30	MEAN
↑ Upstream	River Water	405.6	4	4	5	15	9	8	8
	River Water	404.3	3	3	5	7	9	12	7
	River Water	403.4	4	3	6	6	13	8	6
	Burlington STP Influent	403.2	375	394	278	293	245	310	316
	Burlington STP Effluent	403.2	313	315	160	247	168	234	240
	STP Mixing Zone	403.15	-	-	-	-	35	28	31
↓ Downstream	River Water	403.0	4	6	12	10	12	10	9

TABLE II

Fecal Coliform Density Data

	SAMPLING POINT	RIVER MILEAGE	NUMBER OF FECAL COLIFORMS PER 100 ml 8-27	9-2	9-9	9-16	9-24	9-30	MEAN
Upstream ⟶	River Water	405.6	100	40	124	5000	4700	2700	2110
	River Water	404.3	520	28	101	2100	4600	2200	1592
	River Water	403.4	65	28	84	1450	4800	2100	1421
	Burlington STP Influent	403.2	-	1.12×10^{6}	1.67×10^{6}	1.03×10^{6}	1.13×10^{6}	0.99×10^{6}	1.19×10^{6}
	Burlington STP Effluent	403.2	2×10^{5}	11.1×10^{4}	2.34×10^{5}	1.47×10^{5}	1.58×10^{5}	1.87×10^{4}	1.43×10^{5}
	STP Mixing Zone	403.15	-	-	-	-	1.69×10^{4}	1×10^{4}	1.35×10^{4}
⟵ Downstream	River Water	403.0	380	700	136	2200	9500	1800	2453

FIGURE 1
RELATIONSHIP BETWEEN CONCENTRATION OF COPROSTAN-3-BETA-OL
AND NUMBER OF FECAL COLIFORMS



APPENDIX I

Sample Description

Sample Number	Date Collected	Date Recd.	Time Taken	Sample	River Mileage
First Replicate Series					
7236	8-27-70	8-28-70	1415	Mississippi River	405.6
7237	"	"	1430	"	404.3
7238	"	"	1445	Burlington STP Influent	403.2
7239	"	"	1450	Burlington STP Effluent	403.2
7240	"	"	1515	Mississippi River	403.0
7241	"	"	1525	"	403.4
Second Replicate Series					
7384	9-2-70	9-3-70	1300	Mississippi River	405.6
7385	"	"	1310	"	404.3
7386	"	"	1435	Burlington STP Influent	403.2
7387	"	"	1520	Burlington STP Effluent	403.2
7388	"	"	1335	Mississippi River	403.0
7389	"	"	1345	"	403.4
Third Replicate Series					
7483	9-9-70	9-10-70	0850	Mississippi River	405.6
7484	"	"	0900	"	404.3
7485	"	"	0913	"	403.4
7486	"	"	0942	Burlington STP Influent	403.2
7487	"	"	0955	Burlington STP Effluent	403.2
7488	"	"	0927	Mississippi River	403.0

APPENDIX I (continued)

Sample Number	Date Collected	Date Recd.	Time Taken	Sample	River Mileage
Fourth Replicate Series					
7489	9-16-70	9-17-70	0920	Mississippi River	405.6
7490	"	"	0925	"	404.3
7491	"	"	0935	"	403.4
7492	"	"	0955	Burlington STP Influent	403.2
7493	"	"	1010	Burlington STP Effluent	403.2
7494	"	"	0945	Mississippi River	403.0
Fifth Replicate Series					
7495	9-24-70	9-25-70	0825	Mississippi River	405.6
7496	"	"	0835	"	404.3
7497	"	"	0845	"	403.4
7498	"	"	0915	Burlington STP Influent	403.2
7499	"	"	0930	Burlington STP Ef luent	403.2
7500	"	"	0900	Mississippi River	403.0
7501	"	"	0855	STP Mixing Zone Mississippi River	403.15
Sixth Replicate Series					
7502	9-30-70	10-1-70	0855	Mississippi River	405.6
7503	"	"	0910	"	404.3
7504	"	"	0915	"	403.4
7505	"	"	0945	Burlington STP Influent	403.2
7506	"	"	1000	Burlington STP Effluent	403.2
7507	"	"	0930	STP Mixing Zone Mississippi River	403.15
7508	"	"	0925	Mississippi River	403.0

APPENDIX II

METHODOLOGY

Analysis of Cholesterol and Coprostanol in Rivers and Wastewaters

I. Hexane extraction of sterols and sterol conjugates.

1. To 1 liter samples add 20 ml of conc. HCl and 20 ml of 20% NaCl aqueous solution. Mix thoroughly.
2. Extract three times with 1/5 volumes of prepurified hexane. Extraction is performed in 2 liter capacity separatory funnels on a reciprocal shaker.
3. The pooled hexane fractions are then passed through a column of sodium sulfate to eliminate the water content.
4. The hexane is evaporated in a flash evaporator under vacuum to near dryness.

II. Mild alkali-ethanol hydrolysis of hexane extracted sterols.

1. The near dry residue is transferred to small digestion flasks.
2. Add 3 ml of 25% KOH aqueous solution and mix the contents thoroughly.
3. Add 7 ml of 95% ethyl alcohol and mix the contents thoroughly.
4. Add a few boiling chips to each of the digestion flasks and place them on a hot plate, connect them to the condensers and hydrolyze the contents for 2 hours.
5. After hydrolysis, cool the flasks immediately in an ice bath.

III. Hexane extraction of hydrolysates.

1. To the chilled contents in digestion flasks add 10 ml of distilled water.
2. Transfer the contents to 125 ml capacity separatory funnels.
3. Rinse the digestion flasks thoroughly with 40 ml of prepurified hexane and pour the solvent into the separatory funnels.
4. Extract on reciprocal shaker.
5. Repeat extraction with second 40 ml volume of hexane.
6. The pooled two hexane fractions are rinsed with 10 ml of 2% aqueous NaCl solution, shaken thoroughly, and allowed to stand for 10 minutes.
7. The NaCl layer is drained off and the hexane layer is transferred to evaporation flasks and evaporated in a flash evaporator to almost dryness.

IV. Transfer of hydrolyzed extract to small storage tubes.

1. The near dry residue in evaporation flasks is rinsed 3 times with 2 ml volumes of absolute ethanol, each rinsing facilitated by 10-minute shaking on a reciprocal or rotary shaker.
2. The rinsings are placed into small tubes and the contents are evaporated to dryness under forced prepurified air on a specially designed steam bath.
3. Follow the rinsing of the evaporation flasks with two additional acetone rinsings using 2 ml of acetone for each rinse.
4. Acetone rinsings are evaporated in the tubes, using above method.

V. Preliminary thin layer chromatography of the sterol extracts.

1. 20 by 20 cm glass plates are coated with 0.25 mm layer of Silica Gel G (E. Merck, Darmstadt) and activate for one hour at 110 C.
2. The prepared plates are stored in a vacuum desiccator until used.
3. Standard solutions of the sterols in acetone are prepared as 0.1% solutions, and the reference standards are spotted by means of Hamilton syringes on the Silica Gel G plates, using 5 ug/5 ul twice per each spot.
4. To the hydrolyzed samples of extracted sterols in small tubes are added 0.5 ml volumes of warm acetone. The contents are dissolved in acetone and 10 ul volumes of each of the extract samples are spotted on the same plate next to the reference standards.
5. The drying of the spots is facilitated by the use of infrared lamp.
6. The chromatoplates are developed with a mixture of chloroform and ether in the ratio of 9:1. They are then air dried for 15 minutes and heat dried (110 C) for additional 5 minutes.
7. The chromatoplates are sprayed with a 10% solution of phosphomolybdic acid in 95% ethanol.
8. After heating at 100 C for 15 minutes, the sterols appear as dark spots (violet-blue-indigo) on a yellow background.
9. The Rf values of the standard references are compared with those of the sterol extracts.

VI. Preparative thin layer chromatography for GC chromatography analysis.

1. Reference standards of sterols are spotted on the plate using the same concentrations per spot as above.
2. 0.1 ml volumes of the hydrolyzed samples of extracted sterols in acetone are streaked on the same plates with the standards on the right half portions of each plate.
3. After drying, the chromatoplates are developed with a mixture of $CHCl_3$-ether in the ratio of 9:1, air dried and heat dried.

4. The sterols are located by covering that portion of the plate containing the unknown samples with aluminum foil and spraying the reference standards with 10% solution of phosphomolybdic acid in 95% ethanol.
5. The standard reference spots are visualized after heating at 100 C for 15 minutes.
6. Areas for unknown samples corresponding to locations of reference standards are removed with a micro spatula, placed in prewashed medicine droppers plugged with prewashed glass wool and eluted into micro thistle tubes with 5 ml of acetone.
7. The eluted sterols are concentrated in the thistle tubes to the top level of the thistle on a steam bath with the aid of a gentle stream of clean, dry air.

VII. Gas-liquid chromatography of the silica Gel G eluted sterols.

1. Quantitative determinations are made with an Aerograph gas chromatograph and using a flame ionization detector.
2. A stainless steel column 1/8 in x 6 ft is packed with 60-80 mesh chromasorb Q coated with fluoroalkyl silicone polymer QF-1 (3% by weight) and column temperature is maintained at 235 C.
3. Helium is used as carrier gas, helium pressure adjusted to 25 psi, and the carrier gas flow rate maintained at 48 ml per minute.
4. Hydrogen flow rate to the flame ionization detector is kept at 25 ml/min. and the source of hydrogen is a hydrogen generator.
5. The air flow to the ionization detector is maintained at 200 ml/min.
6. The temperatures of the injector and detector are kept at 240 C.
7. 5 ug/5 ul concentrations of reference standards are injected and standard reference peaks determined for the concentrations of each of the standard sterols injected.
8. The retention time of each of the respective sterol standards is determined for the above set of column and detector conditions used.
9. Quantitative determinations of the unknown sterol extracts are based on peak height compared to a standard curve prepared from known reference standards. Qualitative determinations of unknowns are based on the retention time as compared with that of standards.
10. Internal standards are employed in the analysis of each of the separated (TLC) unknown sterol fractions to definitely categorize the unknown sample.